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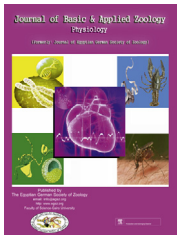

Effect of CoO nanoparticles on the carbohydrate metabolism of the brain of mice "*Mus musculus*"

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KEYWORDS

Cobalt oxide;
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Abstract The effect of CoO nanoparticles (NPs) on the brain of mice administered through gastrointestinal tract for a period of 30 days was studied. AAS analysis revealed that NPs administered orally were retained by cerebellum, cerebral cortex, medulla oblongata and olfactory bulb. This retention of nanoparticles by the brain promoted a significant increase in glucose, pyruvate, lactate and glycogen levels along with the concomitant increase in hexokinase, glucose 6 phosphatase, and lactate dehydrogense activities. However, a decrease in glucose 6 phosphate dehydrogenase activity was observed in the brain regions indicating a deterioration of the pentose phosphate pathway. Thus, the present study suggests that the CoO NPs affect the carbohydrate metabolism of the brain.

Introduction

Cobalt is an important metal, which is used as a binder in the metal industry and as a constituent of most alloys (Jensen and Tüchsen, 1990). Cobalt-based NPs, particularly cobalt oxide NPs are attracting enormous interest currently due to their unique shape and size-dependent properties and are used in different applications like catalysis, magnetism, sensors, electrochemistry, pigments, and energy storage (Liu et al., 2005; Papis et al., 2009). They are one of the interesting chemical compounds used in biomedical applications as a starting material for the construction of dextran coating and magnetic polymer microspheres. In medicine, cobalt is known as an MRI contrast agent in combination with iron, gold, graphite and platinum (Rebello et al., 2010; Magaye et al., 2012).

Cobalt is also used for anaerobic waste water treatment and cancer therapy. Exposure of cobalt to humans occurs mainly from the environment, industry or after joint replacement in implants from the cobalt-chrome alloy. These exposures may lead to numerous lung diseases, including fibrosis, interstitial pneumonitis and asthma (Magaye et al., 2012). The potential of cobalt and its compounds as carcinogenic agent was evaluated by IARC in 1991.

Currently, the application and use of cobalt NPs range from industry to medicine, but research data on its bioeffects are limited. In addition, very little is known about the toxicity of cobalt nanoparticles and it is assumed that its biological activity is mediated by ionic form and can be determined by evaluating its soluble compound as for other metals but evidences showed that the biological activity of cobalt is not exclusively mediated by ionic form dissolved in biological media (Magaye et al., 2012).

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Cobalt nanoparticles are known to be genotoxic *in vitro*. These NPs are internalized by leukocytes, further they interact with DNA, leading to genotoxic effects which are seen on the reticuloendothelial system (Magaye et al., 2012). Further it is reported that cobalt oxide NPs enter the cells, remains confined to vesicles causing production of ROS in human cell lines (Papis et al., 2009). The *in vivo* study carried out in rats revealed that cobalt nanoparticles induced malignant mesenchymal tumors (Magaye et al., 2012). The inhalation study exposed to CoO aerosols with hamsters remained non-positive (Jensen and Tüchsen, 1990) wherein, Syrian golden hamsters exposed to cobalt oxide dusts showed no increase in tumors, however, the study was faulted for poor survival (IARC, 1991). Doses given to the mixed population of rats over 2 years showed bronchio-alveolar proliferation at high rates showing benign lung tumor, bronchio-alveolar carcinoma, adenocarcinomas and bronchioalveolar adenomas (Bucher et al., 1999). These studies indicate that cobalt nanoparticles cause genotoxic and carcinogenic activity *in vitro* and *in vivo* (Magaye et al., 2012). However, a few reports indicate that NPs can reach the brain and are associated with neurodegeneration leading to Parkinson's disease, Huntington's disease, Alzheimer's disease and primary brain tumors (Win-Shwe and Fujimaki, 2011). The exact cause of these diseases is still unknown, but environmental factors or pollutants, including NPs, may be a potential risk factor. NPs can enter the body via different routes like inhalation, dermal penetration, and ingestion, and then are distributed to various tissues including the brain by means of systemic circulation, so it becomes necessary to evaluate the toxic effects of these NPs on the brain (Win-Shwe and Fujimaki, 2011). Thus the review of literature indicates that most of the studies of cobalt effects are with reference to *in vitro* effects showing pseudo-tumor formation, genotoxicity on mammalian cells, peripheral leuckocytes, BALB/3T3 mouse fibroblast cells and macrophages whereas, the *in vivo* studies are only on malignant mesenchymal tumors. However, there is hardly any report of CoO NPs action on the brain as well as their retention and influence on carbohydrate metabolism of the brain. Therefore, here an attempt is made to investigate the action of CoO NPs on carbohydrate metabolism of the brain of mice.

Materials and methods

Nanoparticles

Cobalt oxide nanoparticles measuring average size 50 nm, spherical in shape, black in color, 99% pure, powder in form, without any odor, having specific surface area $\geqslant 10\ m^2/g$, density 6.1 g/cm^3 and with Zeta potential −20.4 mV were obtained from Nanoshel, Wilmington, USA (Product code NS6130-03-380).

Preparation of nanoparticles suspensions

The NPs were suspended directly in mammalian saline (0.9% NaCl prepared with deionized water (DI water)) in order to coat the NPS with saline for their stabilzation and then dispersed by using a sonicator. A stock concentration of 100 mg/10 ml was prepared, further the dilutions were made as per the concentration and body weight of mice. Three concentrations were used as 5 mg, 10 mg and 20 mg/kg body weight (bwt) of mice. The stock and diluted suspensions of CoO NPs were monitored for any agglomeration through microscopic observations of sample drops of stock and suspensions. The shaker bath set at 30 °C helped to maintain suspension of NPs in mammalian saline.

Maintenance of animals

The healthy Mice (*Mus musculus*), weighing 22–32 g were housed in polypropylene cages. The animals were maintained at ambient laboratory conditions, with a dark and light cycle of 12 h having free access to water and standard pellet diet (Hindustan Lever, Bangalore, India). Ethical approval (Ref No. 105/C-2013) was obtained from the Institutional Animal Ethics Committee, based on the CPCSEA guidelines (CPCSEA, 2003) and were followed throughout the study period. The animals were maintained at animal house facility in Department of Zoology, Goa University.

Chronic treatment (30 days)

Mice were divided into 4 groups (5 mice/group) such as Controls, Exp 1(5 mg/kg), Exp 2(10 mg/kg) and Exp 3 (20 mg/kg). Based on the Ld50 values the sub lethal doses were selected for exposure (Shaikh et al., 2015). The animals were fed orally with nanoparticles (suspended in 0.5 ml mammalian saline (0.9% NaCl)) for 30 days (respective dose referred above at 10 am every day). The controls received 0.5 ml of mammalian saline. After thirty days, the animals were anaesthetized using avertin and perfused by mammalian saline. For perfusion a small cut was given at the right atrium and the butterfly needle containing 20 ml of saline was slowly flushed through the left atrium at the rate of 2 ml/min. Further the mice were decapitated and the brain was immediately harvested and transferred to an ice cold mammalian saline (0.9% NaCl). All the brain regions viz., cerebellum, cerebral hemisphere, olfactory bulb, medulla oblongata, hippocampus, cingulate gyrus, pons were quickly isolated aseptically in a cold chamber set at −4 °C. Each isolated region was further cut into tiny pieces and thoroughly and repeatedly rinsed till all traces of blood were removed. Further the tiny pieces were viewed under stereo zoom microscope to ensure that there were no blood traces. Such pieces were once again rinsed thoroughly with cold saline and then immediately stored at −20 °C until use. Assessment of post mortem induced changes to the mice brain was performed as described by Hunsucker et al. (2008) as well as by viewing fresh frozen sections under the microscope.

Deposition of nanoparticles in the brain

After perfusion, the mice were sacrificed by decapitation method, the brain was removed, rinsed with mammalian saline, and its cerebral cortex, cerebellum, medulla oblongata, olfactory bulb, hippocampus, cingulate gyrus, pons were separated carefully as mentioned above. The remaining brain tissue was also carefully rinsed with mammalian saline and stored for detection of CoO NPs at −4 °C until use (hippocampus, cingulate gyrus, pons and remaining brain tissue when separated had CoO NPs below detectable limit, so they were pooled together for detecting CoO NPs). These brain parts along with

Table 1 Retention/deposition of cobalt oxide nanoparticles in the discrete brain regions of the mice.

Doses (CoO NPs) in mg/kg bwt of mice	Total retention of cobalt in the whole brain ($n = 6$)	Brain regions	Concentration in µg/gm dry wt. of tissue	% distribution in the brain
5 mg/kg	49.15 µg/gm dry wt. of tissue	Cerebral cortex (CC)	3.50 ± 0.208	**7.12**
		Cerebellum (C)	9.74 ± 0.376	**19.83**
		Medulla oblongata (MO)	12.61 ± 0.606	**25.66**
		Olfactory bulb (OB)	19.12 ± 1.453	**38.90**
		Hippocampus (H)	ND	–
		Pons (P)	ND	–
		Cingulate gyrus (CG)	ND	–
		Other brain regions + (H, P and CG)	4.16 ± 0.007	8.47
10 mg/kg	96.67 µg/gm dry wt. of tissue	Cerebral cortex	6.51 ± 0.266	**6.95**
		Cerebellum	18.86 ± 0.368	**19.50**
		Medulla oblongata	22.05 ± 0.818	**22.82**
		Olfactory bulb	41.33 ± 2.185	**42.75**
		Hippocampus	ND	–
		Pons	ND	–
		Cingulate gyrus	ND	–
		Other brain regions + (H, P and CG)	7.71 ± 0.009	7.97
20 mg/kg	171.18 µg/gm dry wt. of tissue	Cerebral cortex	11.01 ± 0.271	**6.434**
		Cerebellum	34.51 ± 1.511	**20.16**
		Medulla oblongata	37.68 ± 0.881	**22.02**
		Olfactory bulb	74.12 ± 2.603	**43.30**
		Hippocampus	ND	–
		Pons	ND	–
		Cingulate gyrus	ND	–
		Other brain regions + (H, P and CG)	13.84 ± 0.029	8.08

Bwt – body weight, ND – non detectable, all values are expressed as mean ± SE ($p \leq 0.001$).
Significance level for C, CC, MO, OB and other regions is $P \leqslant 0.001$.

the pooled tissues were then dried in a clean oven completely at 60–70 °C. Each dried brain tissue was digested using 30% hydrogen peroxide followed by concentrated ultra pure nitric acid. The digested ash was then dissolved in 0.25 N nitric acid. Each digested sample was further diluted suitably using 0.25 N nitric acid depending on the type and amount of tissue prior to the analysis (Lasagna-Reeves et al., 2010) The analysis was performed with Atomic absorption spectrophotometer; model no-varian AA 240 FS having Air acetylene flame. Standards were used for all the calibration curves and for calculating metal recovery from tissues standard samples of Bovine Liver (1577b, U.S NIST Gaithersburg, MD 20899) were used (Shaikh et al., 2015). The% distribution/retention in the brain was calculated by using the formula: % retention = retention of cobalt in brain region/retention of cobalt in whole brain × 100.

Sample preparation

The brain regions (cerebellum, cerebral hemisphere, olfactory bulb and medulla oblongata) were thawed and homogenized in 0.32 M sucrose for enzyme assays, for pyruvate assay 0.25 M sucrose was used while for glucose and lactate assays distilled water and 10% Trichloroacetic acid (TCA) were used respectively. The homogenization was done in a cold chamber set at 4 °C. The respective homogenate was centrifuged at 3000 rpm for 15–20 min. The resulted supernatants were stored in clean sterile micro centrifuge tubes at −4 °C until analysis.

Metabolite assays

Glucose: It was estimated by GOD/POD method using commercially available Coral crest biosystem kit (Product No GLU1276).

Pyruvate: To a 0.1 ml of sample, 1 ml 2,4-dinitrophenylhydrazine (DNPH) reagent was added and incubated for 15 min at 37 °C, then 4 N NaOH was added and the mixture was kept in room temperature for 5 mins and OD were taken at 440 nm. Standard pyruvate curve was used to calculate the pyruvate concentration in the samples (Therisa and Desai, 2012).

Lactate: To a 1.0 ml of sample, 1.0 ml 20% $CuSO_4$ solution, 8 ml H_2O and 1 gm of powdered Calcium hydroxide were added and the mixture was kept at room temperature for 30 min. After centrifugation, 0.05 ml of 0.4% $CuSO_4$ was added to the supernatant, followed by 6 ml of Conc. H_2SO_4 and this was kept in a boiling water bath for 5 min. After cooling, 0.1 ml of p-hydroxydiphenyl reagent was added and again the mixture was kept at room temperature for 30 min and then in boiling water bath for 90 s. After cooling the OD was obtained at 560 nm. The lactate concentration was calculated using the lactate standard curve (Therisa and Desai, 2012).

Glycogen: To 1.0 ml of sample, 5 ml of 95% ethanol was added and kept overnight at 4 °C to precipitate. Next day after centrifugation the supernatant was discarded. To the residue 1 ml of distilled water was added and gently mixed, then 4 ml of anthrone reagent followed by Foline ciocalteau reagent was added and the mixture was kept in a water bath for 15 mins and the absorbance was measured at 620 nm.

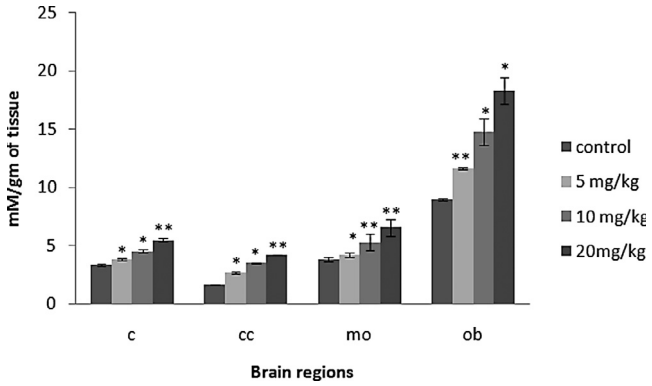

Figure 1 Effect of CoO NPs on the glucose content in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

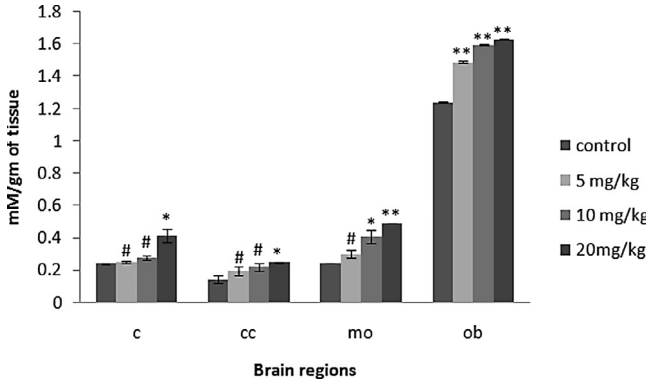

Figure 2 Effect of CoO NPs on the pyruvate content in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

The concentrations were measured using the glycogen standard curve (Caroll et al., 1956).

Protein: Estimated using Lowry's method (Lowry et al., 1952).

Metabolic enzyme assays

Hexokinase (EC 2.7.1.1), glucose 6 phosphate dehydrogenase (EC 1.1.1.49) was estimated following the protocol from Worthington manual (Worthington, 1988), glucose 6 phosphatase (EC 3.1.3.9) was assayed following a protocol of Baginsky et al. (1974), lactate dehydrogenase (LDH) activity was assayed using Mod. IFCC Method by using commercially available Crest Biosystems kit (Product no-LDL1159).

Statistical analysis

All experimental data are expressed as mean ± SE. Statistical analysis of the data was performed using Student's *t*-test (Graph pad Software, San Diego, CA) and a one-way analysis of variance (ANOVA) using XLSTAT software. The criterion for statistical significance was $p \leqslant 0.05$ and for high significance was $p \leqslant 0.001$.

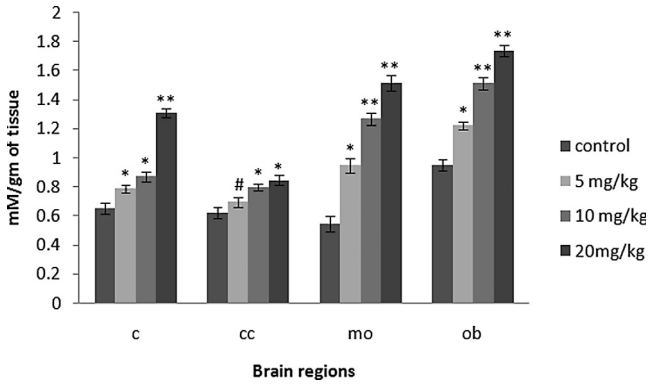

Figure 3 Effect of CoO NPs on the lactate content in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

Results

The procedure adopted to isolate the brain regions and preparation of samples did not promote any postmortem changes in the brain when analyzed for cytoarchitectural changes.

Retention of nanoparticles

The maximum retention of cobalt oxide nanoparticles was seen in cerebral cortex, cerebellum, medulla oblongata and olfactory bulb (Table 1) in comparison with other brain regions. Since the CoO NPs presence was below the detection level in the hippocampus, cingulate gyrus, pons as well as other pooled tissues of the brain, the changes in the carbohydrate metabolism vis-a-vis metabolites and enzymes associated with metabolites for these regions were not studied.

Metabolites

The cerebral cortex, cerebellum, medulla oblongata and olfactory bulb showed a significant dose dependant increase in the concentrations of glucose ($p \leqslant 0.001$), lactate ($p \leqslant 0.05$),

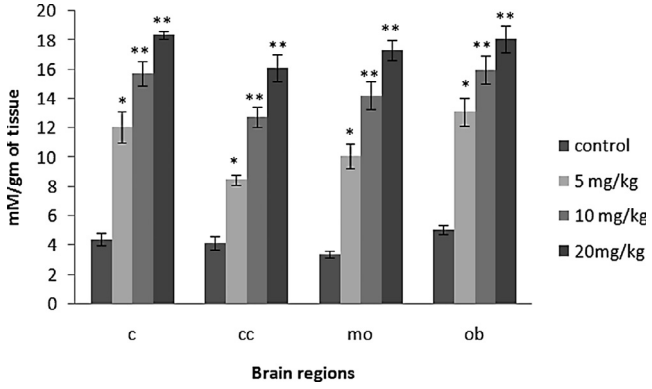

Figure 4 Effect of CoO NPs on the glycogen content in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob-olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

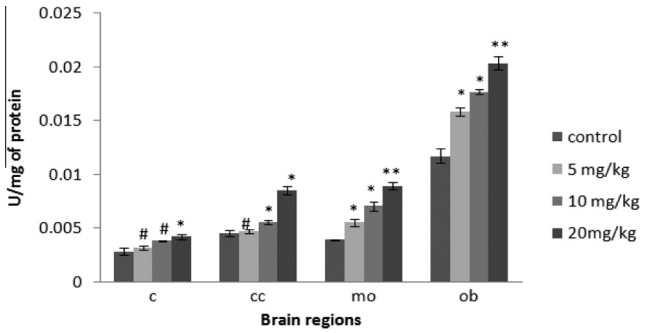

Figure 5 Effect of CoO NPs on the hexokinase activity in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

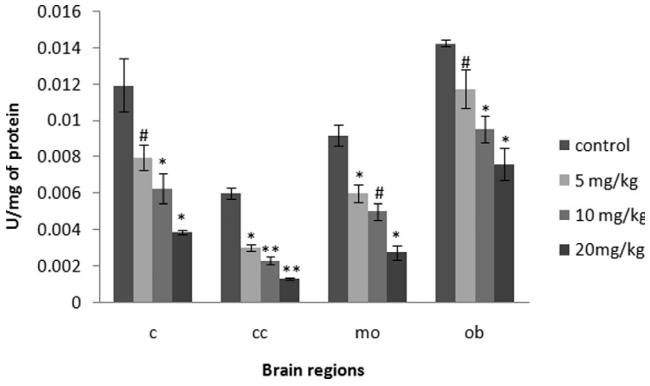

Figure 6 Effect of CoO NPs on the glucose 6 phosphate dehydrogenase activity in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

pyruvate ($p \leqslant 0.05$) and glycogen ($p \leqslant 0.001$) as compared to control mice on exposure to CoO NPs for a period of 30 days(Figs. 1–4).

Enzyme activity

The CoO NPs appear to significantly increase the activities of Hexokinase ($p \leqslant 0.05$), glucose 6 phosphatase ($p \leqslant 0.05$) and lactate dehydrogenase ($p \leqslant 0.05$) dose dependently in all the aforementioned regions of the brain whereas glucose 6 phosphate dehydrogenase activities decreased significantly ($p \leqslant 0.05$) as compared to controls(Figs. 5–8).

Discussion

The effect of cobalt oxide nanoparticles on the mice in terms of body weight and organ index, as well as distribution and retention of nanoparticles in different vital organs of body including brain is already reported by us earlier (Shaikh et al., 2015). It is hypothesized that environmental pollutants including nanoparticles might be one of the reasons for neurodisorders, but there are not many experimental evidences to substantiate

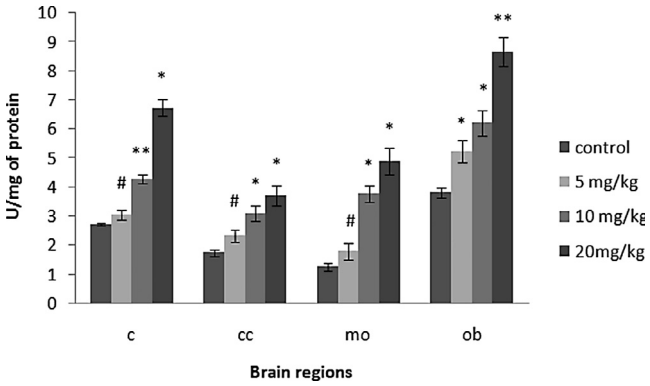

Figure 7 Effect of CoO NPs on the glucose 6 Phosphatase activity in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

it (Win-Shwe and Fujimaki, 2011). Here we report the effect of CoO NPs on the brain. The mice fed with CoO NPs showed the presence of these NPs in the brain indicating its retention in it. Since the isolated brain pieces were divested of any traces of blood, the entry of nanoparticles into the brain indicates its entry into the neural tissue either through leaky passages of the blood vessel's endothelium reaching the brain or through compromising the blood–brain barrier. Among all the regions of the brain tested for the presence of CoO NPs, their retention was maximum in the cerebral cortex, cerebellum, medulla oblongata and olfactory bulbs as these four regions are most likely to come in contact with the nanoparticles after they cross the blood–brain barrier and enter the brain. Therefore, the chances of the nanoparticles retention/deposition in these regions are high.

Carbohydrate metabolism is one of the important biochemical fundamental processes that takes care of continuous supply of energy to living cells and helps in normal functioning of the body. The mice brain exposed to CoO NPs showed a significant dose dependant increase in glucose levels which may promote atrophy of the brain as observed by Cherbuin et al. (2012) and McMillen (2012) in the hippocampus and such

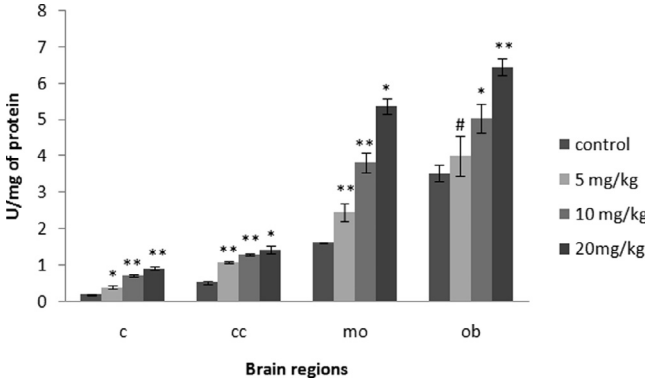

Figure 8 Effect of CoO NPs on the LDH activity in the brain of mice (c – cerebellum, cc – cerebral cortex, mo – medulla oblongata, ob – olfactory bulb, (* – $P \leqslant 0.05$, ** – $P \leqslant 0.001$, # – non-significant compared to controls)).

atrophy can seriously affect the functions associated with cerebral cortex, cerebellum, medulla oblongata, olfactory bulb and even other brain regions. The brain requires two times more energy than the other tissue cells and due to stress created by the retention of CoO NPs, the demand of glucose by the brain could elevate leading to stepping up of carbohydrate metabolism to mitigate glucose demand by the affected regions of the brain. Besides the astrocytes and astroglial cells of the brain could be contributing to an increase in glucose levels of the brain by the breakdown of glycogen stored in them. Astrocytes and astroglial cells are known as sources of glycogen in the brain (Belanger et al., 2011). A further increase in the glucose 6 phosphatase activity conveys that the elevated glucose levels are also due to formation of glucose from the non carbohydrate substrates via gluconeogenesis as suggested by Gerich et al. (2001), Garrett and Charles (2002) in non neural tissues. Also in mammalian tissues glucose 6 phosphate may return to glucose via phosphatase action or be used in the synthesis of glycogen or may enter into the energy yielding Embeden Meyerhof pathway or into the 6 phosphogluconate pathway (Irwin and Edward, 1964). Therefore, it is likely that elevation of glucose 6 phosphatase could be an attempt of the brain tissue to divert excessive glucose 6 phosphate either to glucose or to the process of glycogen synthesis or to divert it to energy yielding pathways mentioned earlier to mitigate energy demand.

Hexokinase is the first enzyme involved in glucose metabolism and is the major metabolic regulatory step in the brain. Its activity is complex because more than 80% of brain hexokinase is bound to the cell organelles which profoundly alter the kinetics of the enzyme (Bigl et al., 1999). Damage to the brain or stress to the neurons could lead to an increase in hexokinase activity as an attempt to step up metabolism to meet the energy demand and increased hexokinase activity can lead to an increase in pyruvate levels on the one hand and glycogen on the other. Elevation of lactate dehydrogenase was also observed in all regions of the brain under study indicating damage caused by CoO NPs to the brain (Pulliam et al., 1991) giving rise to an increase in lactic acid formation. The accumulation or rise in lactate levels of the brain could be due to elevated pyruvate levels as well as elevated LDH activity which breakdowns pyruvate to lactate. Lactate is formed in the brain as an energy source and it is known that this lactate triggers the release of non adrenaline by neurons to mitigate neuronal stress (Tang et al., 2014).

Interestingly wherein all the metabolites and enzyme levels were found to be increasing there is one enzyme that showed completely opposite behavior i.e. glucose 6 phosphate dehydrogenase which showed a significant decrease in the brain regions affecting the pentose phosphate pathway. Glucose 6 phosphate dehydrogenase has an important function in intermediary metabolism since it catalyzes the very first step in the pentose phosphate pathway and also provides reductive potential in the form of NADPH. This enzyme is conventionally referred to as first and rate limiting enzyme of the pentose phosphate pathway (Pandolfi et al., 1995). Therefore a drop in glucose 6 phosphate dehydrogenase activities in all parts of the brain indicate a decrease in the pentose phosphate pathway as well as a decrease in NADPH and also a decrease in defense against oxidative stress. This change in the activity of the enzyme system depends upon the dose and duration of nanoparticle exposure as well as on the susceptibility of disturbed neuronal metabolism (Uttara et al., 2009). This clearly indicates that CoO NPs cause neural stress and disturbance in the exposed animal (Bano and Bhatt, 2010) and stepping up of carbohydrate metabolism of the brain. However, it is not possible to know if the changes in brain biochemistry reported here were primary toxic effects of the cobalt nanomaterials or a secondary consequence of systemic toxicity.

Conclusion

Chronic gastrointestinal exposure of mice to CoO NPs promotes its retention by the brain, especially by cerebral cortex, cerebellum, medulla oblongata and olfactory bulb leading to changes in the carbohydrate metabolism of the brain.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

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